ERR1 and PGC1α associated mitochondrial alterations correlate with pan-cancer disparity in African Americans

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BACKGROUND. African American (AA) patients have higher cancer mortality rates and shorter survival times compared to European American (EA) patients. Despite a significant focus on socioeconomic factors, recent findings strongly argue the existence of biological factors driving this disparity. Most of these factors have been described in a cancer-type specific context rather than a pan-cancer setting.

METHODS. A novel in silico approach based on Gene Set Enrichment Analysis (GSEA) coupled to Transcription Factor enrichment was carried out to identify common biological drivers of pan-cancer racial disparity using The Cancer Genome Atlas (TCGA) dataset. Mitochondrial content in patient tissues was examined using a multi-cancer tissue microarray approach (TMA).

RESULTS. Mitochondrial oxidative phosphorylation was uniquely enriched in AA tumors compared to EA tumors across various cancer types. AA tumors also showed strong enrichment for the ERR1-PGC1α-mediated transcriptional program, which has been implicated in mitochondrial biogenesis. TMA analysis revealed that AA cancers harbor significantly more mitochondria compared to their EA counterparts.

CONCLUSIONS. These findings highlight changes in mitochondria as a common distinguishing feature between AA and EA tumors in a pan-cancer setting, and provide the rationale for the repurposing of mitochondrial inhibitors to […]

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ERR1 and PGC1α associated mitochondrial alterations correlate with pan-cancer disparity in African-Americans.

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ABSTRACT

Background: African American (AA) patients have higher cancer mortality rates and shorter survival times compared to European American (EA) patients. Despite a significant focus on socioeconomic factors, recent findings strongly argue the existence of biological factors driving this disparity. Most of these factors have been described in a cancer-type specific context rather than a pan-cancer setting.

Methods: A novel in silico approach based on Gene Set Enrichment Analysis (GSEA) coupled to Transcription Factor enrichment was carried out to identify common biological drivers of pan-cancer racial disparity using The Cancer Genome Atlas (TCGA) dataset. Mitochondrial content in patient tissues was examined using a multi-cancer tissue microarray approach (TMA).

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Conclusions: These findings highlight changes in mitochondria as a common distinguishing feature between AA and EA tumors in a pan-cancer setting, and provide the rationale for the repurposing of mitochondrial inhibitors to treat AA cancers.
INTRODUCTION

African American (AA) cancer patients have higher mortality rates and shorter survival times compared to European American (EA) patients (1–5). In the US, for every 100,000 cancer patients, the age-adjusted cancer-associated mortality for AA versus EA patients was 189.54 and 163.54 respectively, resulting in a disparity ratio of 13.87 % (2–5). When examined for individual tumor types, the disparity in mortality was higher in AA patients for cancers of the breast, colon, rectum, uterus, liver, lung, bronchus, and prostate with disparity ratios ranging from 6.41% (for lung cancer across both genders) to 118.52 % (for men with prostate cancer, PCa). This observation is consistent with the five and ten year survival data obtained from the Surveillance Epidemiology and End Results database (Supplementary Table 1). The specific reasons for this disparity have not been identified. However, genetic/biologic, environmental and health care access/utilization factors are all thought to be contributory(6).

Recent studies suggest that biological factors can be crucial for racial inequalities in cancer incidence and clinical outcomes(7,8). For example, aggressive triple negative breast cancer (BRCA) is more prevalent in AA women and is associated with higher levels of resistin, interleukin 6(9), and 2-hydroxyglutarate(10). AA PCa has higher levels of 17β-estradiol(11), prostate-specific antigen(12), androgen receptor expression(13), and altered mitochondria(14). AA patients with hepatocellular carcinoma have a 7-fold increase in apolipoprotein 1 (APOA1) expression and reduction in hepatocyte nuclear factor 4α (HNF4α), with the latter being associated with increased metastasis(15). Furthermore, AA non-small cell lung cancer patients have higher circulating levels of IL-1β, interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) compared to EA patients(16). Taken together, although these findings demonstrate independent disease-site specific biological alterations between AA and EA patients, they fail to explain the pan-cancer disparity in tumor progression and clinical outcomes observed between these two populations. In light of this, our main goal was to examine if there exist common biological alterations across multiple cancers distinguishing AA and EA tumors.
RESULTS

We performed Gene Set Enrichment Analysis (GSEA) for 23 different cancer types independently using The Cancer Genome Atlas (TCGA) gene expression data, looking for common hallmark alterations capable of distinguishing AA and EA tumors across multiple cancer types (Figure 1A). These 23 cancer data sets contained data on at least 10 AA tumors. GSEA revealed positive enrichment for concepts including oxidative phosphorylation (OXPHOS), DNA repair, and G2M checkpoint in AA versus EA tumors across multiple cancer types (Figures 1A, 1B, Supplementary Table 2, P<0.01, False discovery rate (FDR) < 25%). Co-enrichment of DNA repair and the G2M checkpoint is not surprising as both are related to preserving DNA fidelity(17). OXPHOS is associated with mitochondria and energy production, and the generation of DNA damaging reactive oxygen species (ROS)(18). To validate these initial findings, we performed GSEA on five independent datasets (GSE37751- BRCA, GSE64331- PCa, GSE6956- PCa, GSE101929- Lung cancer, GSE28000- Colon cancer) and found a similar trend for OXPHOS enrichment in AA tumors compared to EA tumors (Figures 1C, 1D, and Supplementary Table 3). Analysis of a subset of genes encoding different mitochondrial complexes within the enriched OXPHOS pathway demonstrated that these were ubiquitously upregulated in AA within several tumor types (Figure 2, Supplementary Table 4, refer to Supplementary Figure 1 for heat map containing top 50 % genes associated with OXPHOS concept). In light of the above, we hypothesized that there exist common transcriptional regulators regulating the OXPHOS gene set in AA tumors across different cancer types.

To identify common transcriptional regulators of the OXPHOS gene set, we performed transcription factor enrichment analysis on each of the 23 cancer datasets. Among the different transcription factors identified within each cancer type (representative example for BRCA in Figure 3A), Estrogen-Related Receptor 1 (ERR1) was the most significant and commonly enriched transcription factor in AA tumors across all the 23 cancer types (Figure 3B, P< 0.0001,
ERR1 is known to regulate mitochondrial biogenesis in the presence of Peroxisome proliferator-activated receptor Gamma Coactivator 1 alpha (PGC1α)(19). Indeed, we found that the 23 cancer types were also co-enriched for PGC1α (Figure 3C). Additionally, there was a significant positive correlation between ERR1 and PGC1α for these cancer types (Figure 3D, Supplementary Table 6, P=0.02, Coefficient of correlation (R) = 0.47). Collectively, our findings suggest that AA tumors are defined by elevated OXPHOS coupled to ERR1 and PGC1α gene set enrichment.

Given that ERR1 and PGC1α together have been shown to regulate mitochondrial biogenesis, and that OXPHOS is a product of mitochondrial respiration, we hypothesized that tumors of AA patients would possess more mitochondria compared to EA patients. To test this hypothesis, we performed tissue microarray analysis (TMA) on a cohort of PCa, laryngeal and oral cancer samples, staining for mitochondria. AA samples stained significantly higher for mitochondria than EA samples in all the three cancer types (Figure 4; refer Supplementary Tables 7-9 for clinical data associated with the TMAs).
DISCUSSION

Our data suggest that AA tumors predominantly express more mitochondria, ERR1, and PCG1α in multiple cancer types. These findings could form the biological basis of disparity in pan-cancer clinical outcomes seen in AA patients. Interestingly, clinical trial data for metformin, a mitochondrial inhibitor, has shown that AA patients, in general, respond better to this drug than EA patients (20). Metformin was also more effective in reducing the incidence of PCa and the risk of colorectal cancer death in AA patients compared to EA patients (21,22). These findings provide a rationale for evaluating existing mitochondrial drugs to treat AA tumors. Additionally, from a biomarker perspective, validation of our results could lead to the development of mitochondrial metabolites as non-invasive biomarkers for cancer prognosis. Although this is the first report to demonstrate the existence of a common biological alteration in AA tumors in a pan-cancer setting, there is a limitation that should be addressed in future studies. In our study, the stratification of patients into AA or EA groups in the TCGA data was solely based on self-reported information associated with the clinical files accompanying these data sets. It is essential to confirm these results using ancestry verified AA tumor data. We have addressed this partially by using ancestry verified PCa TMA that confirms increased mitochondria in AA tumors relative to EA tumors. Irrespective of this caveat, we expect our studies to motivate mechanistic studies focused on mitochondria using ancestry verified AA pre-clinical models.
METHODS

Gene Set Enrichment Analysis (GSEA)

To characterize biologically relevant changes in molecular signaling pathways between AA and EA patients, we employed GSEA (23) to identify significantly enriched concepts in each of the 23 tumor types in TCGA, each of which contained data from at least 10 AA patients. The procedure for GSEA involves determining whether a pre-defined set of genes (e.g., genes involved in a particular molecular signaling pathway) is significantly different between any two groups. The entire list of genes is ranked according to expression differences between two experimental conditions. An enrichment score for each gene set is then calculated. This score represents the extent of overrepresentation of a gene set at either end of this continuum. In GSEA, the cumulative distribution function was constructed by performing 1,000 random gene set membership assignments. A nominal P<0.01 and a false discovery rate (FDR)<25% were used as thresholds for determining the significance of the Enrichment Score (ES). The methodology works in synchrony with the Molecular Signatures Database (MSigDB), which provides the gene set definitions in the form of eight major collections (13,311 total gene sets). Out of the 8 gene set collections, we focused on well-defined, large scale biological processes termed the Hallmark (H) Gene Set. For the GSEA, self-reported data on race was used to stratify TCGA samples into AA and EA groups.

Enrichment Analysis to identify key transcription factors regulating the OXPHOS gene cluster

The OXPHOS gene cluster was defined as the set of core genes that contributed to the enrichment of the OXPHOS pathway in GSEA. We performed an enrichment analysis using the hypergeometric method to identify transcription factors motifs (TFT, C3) in the OXPHOS gene set derived from the MSigDB for each cancer type. A nominal P<0.0001 and an FDR <0.01% were used as thresholds to determine the significance of the enrichment. The results were represented using heat maps and bar graphs implemented using the R package.
Tissue Microarray (TMA) Analysis

The PCa TMA used for mitochondrial staining was built by the Pathology and Histology Core at the Baylor College of Medicine. This TMA comprised 53 stainable ancestry-verified AA tissue sections and 51 stainable EA tissue sections. Laryngeal and oral cancer TMAs containing samples from self-reported AA patients were obtained from Dr. Vlad C. Sandulache and Dr. Andrew Sikora. The laryngeal cancer TMA comprised 11 stainable AA tissue sections and 18 stainable EA tissue sections. The oral cancer TMA comprised 14 stainable AA tissue sections and 43 stainable EA tissue sections.

The tissues were deparaffinized using xylene and rehydrated using a graded alcohol series. The slides were pressure-cooked for 10 minutes to retrieve mitochondrial antigens. The slides were then treated with 3% hydrogen peroxide for 5 minutes to quench endogenous peroxidase activity. Slides were blocked with 3% goat serum at room temperature for one hour in a humidity chamber before staining with an anti-mitochondrial antibody (1:100 dilution of mouse monoclonal antibody, Abcam ab92824). The secondary antibody was applied for 40 minutes (HRP-conjugated goat anti-mouse secondary antibody, Jackson Immunoresearch Laboratories Inc., West Grove, PA). Diaminobenzidine was applied for 7 minutes to visualize the antigen-antibody reaction, and the slides were counterstained with hematoxylin for 1 minute. Each staining run included positive and negative controls; negative controls were obtained by omitting the primary antibody. Slides were then dehydrated in an alcohol series and cleared in xylene baths before being mounted with Permount media. The PCa TMA was scored by Dr. Michael Ittmann, a Genitourinary Pathologist. The laryngeal and oral cancer TMAs were scored by Dr. Wendong Yu, a Head and Neck cancer Pathologist. The TMA staining was determined using a combination of an intensity and extent score. These two values were then multiplied to generate a final score that was used to make the box plots.
P values for the enrichment analyses were generated through the GSEA permutation test (1000 permutations). The FDR was determined using the Benjamini-Hochberg procedure (24). Significance for the mitochondrial TMA analysis was determined using a two-tailed Wilcoxon rank sum test. A P value < 0.05 was considered significant.

**STUDY APPROVAL**

The use of all human tissues in this study was reviewed and approved by the Institutional Review Board at Baylor College of Medicine.
AUTHOR CONTRIBUTIONS

DWBP and ASK formulated and designed the study. DWBP, JMA, and AB performed the bioinformatics analyses. JMA, AB, and SML wrote the manuscript. AB performed the \textit{in vitro} studies. SML analyzed TMA staining data. BK performed TMA staining. PC provided TMA slides. MMI and WY performed TMA scoring. NP and NN provided scientific input. JAJ performed clinical sample annotation. VCS and AS provided the TMAs. GM reviewed the statistical analyses. All authors reviewed the manuscript.
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Figure 1. Molecular concepts significantly enriched in self-reported African American (AA) tumors compared to European American (EA) tumors in pan-cancer GSEA analysis. A) Heatmap showing the top 20 commonly enriched pathways in AA tumors compared to EA tumors, across multiple cancers. See Normalized Enrichment Score (NES) scale on the top. All GSEA concepts listed are significant at FDR<0.25. B) Table showing the individual NES for the three top commonly enriched pathways for each cancer type (FDR<0.05). C) Heatmap showing enrichment of OXPHOS in five independent cancer datasets. D) Table showing individual NES for the top GSEA Concepts.
Figure 2. Genes associated with oxidative phosphorylation are elevated in AA tumors relative to EA tumors across 23 cancer types in TCGA. Log fold change in expression of the genes between AA and EA tumors is shown. Shades of yellow and blue describe increased and reduced fold change in AA vs. EA comparison, respectively. Columns contain different cancer types, and rows contain the genes. Genes are grouped based on their membership in the five different mitochondrial complexes.
Figure 3. OXPHOS gene cluster in African American (AA) tumors enriches for ERR1 and PGC1α transcription factor motifs. A) List of transcription factor motifs enriched by the OXPHOS cluster in breast cancer (BRCA). The significance of the enrichment is described in the X-axis. Y-axis describes the different transcription factor motifs. ERR1 is the most enriched transcription factor motif in the OXPHOS cluster in breast cancer. B) Estrogen-Related Receptor 1 (ERR1) is significantly enriched in OXPHOS gene clusters across multiple cancers. The significance of the enrichment is described in the X-axis. Y-axis describes the different cancer types. C) Same as in B, but for Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). D) A significant positive correlation is observed between ERR1 and PGC1α expression in AA tumors that enriched for OXPHOS pathway across multiple cancers.
Figure 4: Tissue Microarray analysis shows increased mitochondria in African-American patient samples. A) Tissue Microarrays (TMA) stained for mitochondria showed increased mitochondrial staining in African-Americans (AA) compared to B) European-Americans (EA) (representative image). C) TMA data showing increased staining for mitochondria in AA prostate cancer (n=53) compared to EA prostate cancer (n=51). D) Same as in C, but for laryngeal cancer (AA: n=12, EA: n=17). E) Same as in C, but for oral cancer (AA: n=14, EA: n=43) (**P<0.01, *P<0.05 - Wilcoxon rank sum test, data represent Mean ± Standard Deviation).